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Nonequilibrium Kinetics of a Cyclic GMP-binding Protein in *Dictyostelium discoideum*

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ABSTRACT Chemoattractants added to cells of the cellular slime mold *Dictyostelium discoideum* induce a transient elevation of cyclic GMP levels, with a maximum at 10 s and a recovery of basal levels at ~25 s after stimulation. We analyzed the kinetics of an intracellular cGMP binding protein in vitro and in vivo. The cyclic GMP binding protein in vitro at 0°C can be described by its kinetic constants $k_1 = 2.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 3.5 \times 10^{-3} \text{ s}^{-1}$, $K_d = 1.4 \times 10^{-9} \text{ M}$, and 3,000 binding sites/cell. In computer simulation experiments the occupancy of the cGMP binding protein was calculated under nonequilibrium conditions by making use of the kinetic constants of the binding protein and of the shape of the cGMP accumulations. These experiments show that under nonequilibrium conditions the affinity of the binding protein for cGMP is determined by the rate constant of association (k_1) and not by the dissociation constant (K_d). Experiments in vivo were performed by stimulation of aggregative cells with the chemoattractant cAMP, which results in a transient cGMP accumulation. At different times after stimulation with various cAMP concentrations, the cells were homogenized and immediately thereafter the number of binding proteins which were not occupied with native cGMP were determined. The results of these experiments in vivo are in good agreement with the results of the computer experiments. This may indicate that:

- (a) The cGMP binding protein in vivo at 22°C can be described by its kinetic constants: $k_1 = 4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = 6 \times 10^{-3} \text{ s}^{-1}$.
- (b) Binding of cGMP to its binding protein is transient with a maximum at about 20–30 s after chemotactic stimulation, followed by a decay to basal levels, with a half-life of ~2 min.
- (c) The cGMP binding proteins get half maximally occupied at a cGMP accumulation of $\Delta[\text{cGMP}]_{10} = 2 \times 10^{-8} \text{ M}$, which corresponds to an extracellular stimulation of aggregative cells by 10^{-10} M cAMP.
- (d) Since the mean basal cGMP concentration is $\sim 2 \times 10^{-7} \text{ M}$, the small increase of cGMP cannot be detected accurately. Therefore the absence of a measurable cGMP accumulation does not argue against a cGMP function.
- (e) There may exist two compartments of cGMP: one contains almost all the cGMP of unstimulated cells, and the other contains cGMP binding proteins and the cGMP which accumulates after chemotactic stimulation.
- (f) From the kinetics of binding, the cellular responses to the chemoattractant can be divided into two classes: responses which can be mediated by this binding protein (such as light scattering, proton extrusion, PDE induction, and chemotaxis) and responses which cannot be (solely) mediated by this binding protein such as relay, refractoriness, phospholipid methylation, and protein methylation.

The cellular slime mold *Dictyostelium discoideum* lives in the soil where it feeds on bacteria. After exhaustion of the food supply the amoebae pass a transient phase, followed by cell

aggregation and formation of a fruiting body consisting of dead stalk cells and spores embedded in a slime droplet on top of them. Aggregation is mediated by chemotaxis to cAMP (20).

Pulse-wise addition of cAMP to *D. discoideum* cells evokes several responses. In postvegetative cells, cAMP induces the following: a decrease in light absorption in cell suspensions (12), a small, but significant excretion of cAMP, which is involved in the relay of the signal (8), chemotaxis (4), induction of phosphodiesterase (17, 18), and under some conditions, (23) acceleration of development to the aggregative phase (5, 11). In aggregative cells, cAMP induces the following: light scattering (12), relay (8, 14), chemotaxis (20), cGMP accumulation (25, 42), excretion of protons (22), protein methylation (28), and phospholipid methylation (1, 28). The cAMP signal enters the cell via cell surface receptors (15, 16, 21).

All known chemoattractants induce a transient elevation of cGMP levels: cAMP in aggregative cells of *D. discoideum* (25, 42); folic acid in vegetative cells of *D. discoideum* (31, 42) and *D. lacteum*, *D. minutum*, and *Polysphondylium violaceum* (Kakebeeke, personal communication); and partly purified, active extracts (30, 41) which attract specifically *D. lacteum* (24) and *Polysphondylium violaceum* (40).

cAMP added to vegetative cells of *D. discoideum* seems to be an exception; it is chemotactically active at high concentrations (4) but does not induce a measurable elevation of cGMP levels (25). In this paper we will give evidence that this discrepancy does not argue against a cGMP function during chemotaxis.

cGMP probably exerts its function(s) by activating intracellular cGMP binding proteins.

cGMP binding proteins which have been shown in several organisms are often not associated with protein kinase activity (see reference 10). Also, the cGMP binding activity in homogenates of *D. discoideum* cells seems not to be associated with cGMP dependent protein kinase activity (33, 35) and can be divided into at least three fractions with different molecular weights (33). The dissociation constants are $\sim 10^{-8}$ M (32, 33). The mean cGMP concentration in unstimulated *D. discoideum* cells is $\sim 2 \times 10^{-7}$ M (25) which is two orders of magnitude above the dissociation constant. At 2×10^{-7} M, 99% of the cGMP binding proteins should be occupied. This suggests either compartmentalization of cGMP and cGMP binding proteins or differences in the magnitude of the dissociation constant between the in vitro and in vivo states.

The increase of cGMP levels is only transient (24, 25, 26, 31, 40, 42), by which equilibrium of binding will not be reached. To be able to understand the function(s) of the cGMP binding proteins it is necessary to determine the rate constants of association and dissociation and to combine these with the shape of the curve of cGMP accumulation after chemotactic stimulation.

In this paper we describe the determination of the rate constant of association (k_1) and the rate constant of dissociation (k_{-1}) in vitro. Computer simulations of the cGMP binding in vivo were performed by making use of these kinetical data and the shape of the cGMP peak after chemotactic stimulation (25, 31). The predictions of the computer simulations are in close agreement with the results of cGMP binding experiments in vivo. The consequence for a possible function of cGMP will be discussed.

MATERIALS AND METHODS

Culture Conditions

Dictyostelium discoideum, NC-4(H), was grown on SM-agar in association with *Escherichia coli* B/r (19). Cells were harvested in 10 mM sodium-potassium phosphate buffer, pH 6.0 and were freed from bacteria by repeated washing and

centrifugation at 100 g for 4 min. Cells were starved by shaking in 10 mM phosphate buffer 6.0 at a density of 10^7 cells/ml at 22°C.

Homogenate

Cells were starved for 2 h, washed twice in 5 mM Tris/HCl, pH 7.5, and suspended at a density of 2×10^8 cells/ml in Tris/HCl, pH 7.5. Cells were homogenized at 0°C by sonic disruption with a Branson B-12 sonifier with microtip for 3 times 5 s at 50 W. The homogenate was centrifuged at 0°C for 10 min at 30,000 g and the supernatant was centrifuged for 1 h at 48,000 g. The 48,000 g supernatant was used for the binding experiments in vitro which are slightly modified (39) from the procedure described by Mato et al. (32).

Rate Constant of Association

The incubation mixture at 0°C contained 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4 , 2 mM DTT (Sigma Chemical Co., St. Louis, MO), different concentrations of [^3H] cGMP (15 Ci/mmol, Amersham Corp., Arlington Heights, IL) and 48,000 g supernatant in a total volume of 1,750 μl . The incubation was started by the addition of 700 μl of 48,000 g supernatant.

At intervals, 200- μl samples were filtered over Millipore filters (diameter 24 mm, pore size 0.45 μm , Millipore Corp., Bedford, MA). In all experiments, filters were washed twice with 4 ml of 50 mM phosphate buffer pH 6.5 and transferred to 4 ml of Instagel (Packard Instrument Co., Downers Grove, IL); radioactivity was determined with an LKB 1215 rack beta liquid scintillation counter (LKB Instruments, Inc., Rockville, MD). Aspecific binding was determined by including 10^{-5} M cGMP in the incubation mixture.

Rate Constant of Dissociation

The incubation mixture at 0°C contained 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4 , 2 mM DTT, 5×10^{-8} M [^3H]-cGMP (15 Ci/mmol) and 48,000 g supernatant in a total volume of 1,500 μl . The incubation was started by addition of 600 μl of 48,000 g supernatant. About 5 min after the addition of 48,000 g supernatant, equilibrium between association and dissociation was almost reached and further association between [^3H]-cGMP and the binding protein was prevented either by a 50-fold dilution of the incubation mixture in 50 mM phosphate buffer, 3 mM MgSO_4 , 2 mM DTT with or without 10^{-6} M unlabeled cGMP, or by the addition of 15 μl of unlabeled cGMP (10^{-5} M final concentration) to the incubation mixture. Dissociation of ^3H -cGMP from the complex was followed by filtration of 10-ml samples at different times after dilution or by filtration of 200- μl samples at different times after the addition of excess unlabeled cGMP.

In Vivo Experiments

Cyclic GMP levels are temporarily elevated after chemotactic stimulation. This cGMP may be temporarily bound to cGMP receptors. The number of receptors that were not occupied with native cGMP was determined by fast homogenization and immediate incubation with ^3H -cGMP. The homogenate should be incubated long enough with ^3H -cGMP to reach equilibrium but as short as possible since the native cGMP binding protein complex will dissociate; 2×10^{-8} M ^3H -cGMP is sufficient to reach 95% of the equilibrium binding within 1 min and low enough to keep relative aspecific binding small. The partial dissociation of the native complexes will be taken into account during the interpretation of the results. The cGMP content of the cell will compete with ^3H -cGMP for binding to the protein. To keep this competition constant, cells were homogenized after the cGMP accumulation has returned to basal levels and in the presence of DTT at 0°C which inhibits the hydrolysis of native cGMP.

The procedure is as follows: Cells were starved for 5 h, centrifuged, washed twice, and suspended in 10 mM phosphate buffer, pH 6.0, at a density of 2×10^8 cells/ml. Air was bubbled through the suspension for at least 10 min. At $t = 0$ s, cell suspensions (500 μl each) were stimulated at 22°C with 100 μl of different cAMP concentrations. At $t = 10$ s, a 100- μl sample was withdrawn and added to 100 μl of 3.5% (vol/vol) perchloric acid for the determination of the cGMP content (for procedure, see reference 38). 7 s before homogenization was started, 10 μl of DTT (2 mM final concentration) was added and, 2 s later, tubes were placed in ice. Cells were homogenized in ice by sonication for 6 s. 10, 12, and 14 s after homogenization was started, 100- μl homogenates were added to tubes (at 0°C) containing 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4 , 2 mM DTT, 2×10^{-8} M [^3H]-cGMP (final concentrations) in a total volume of 250 μl . 70, 72, and 74 s after the beginning of the homogenization, 200- μl samples were filtrated. Cells were stimulated with different cAMP concentrations and homogenized at different times after stimulation.

RESULTS

Binding of cyclic GMP to a receptor can be given as (see reference 3)



where F is the free receptor, B is the occupied receptor, k_1 is the rate constant of association, and k_{-1} is the rate constant of dissociation.

The increase of the fraction of receptors occupied (b) is given by

$$\frac{db}{dt} = k_1[\text{cGMP}](1 - b) - k_{-1}b \quad (2)$$

Association

The binding of cGMP to the binding protein before and during the establishment of equilibrium is shown in Fig. 1A. Assuming that there are far less cGMP binding proteins than cGMP molecules, Eq. 2 is solved yielding:

$$-\ln\left(1 - \frac{b}{x}\right) = (k_1[\text{cGMP}] + k_{-1})t \quad (3)$$

where x is the fraction of receptors occupied at equilibrium. Representation of the data of Fig. 1A as the left part of Eq. 3 vs. time gives straight lines (Fig. 1B) with a slope (α) which equals

$$\alpha = k_1[\text{cGMP}] + k_{-1} \quad (4)$$

Presentation of α vs. the cGMP concentration results in a straight line (Fig. 1C) with a slope equal to k_1 , an intercept on the ordinate equal to k_{-1} , and an intercept on the abscissa equal to $-K_d$. The experiment shown in Fig. 1 gives the following constants: $k_1 = 1.8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 2.3 \times 10^{-3} \text{ s}^{-1}$, and $K_d = 1.4 \times 10^{-9} \text{ M}$.

Dissociation

The rate constant of dissociation (k_{-1}) can be calculated from experiments in which association between ^3H -cGMP and the binding protein is prevented after equilibrium is reached. If new associations of ^3H -cGMP do not take place, the following equation can be derived:

$$-\ln \frac{b}{y} = k_{-1}t \quad (5)$$

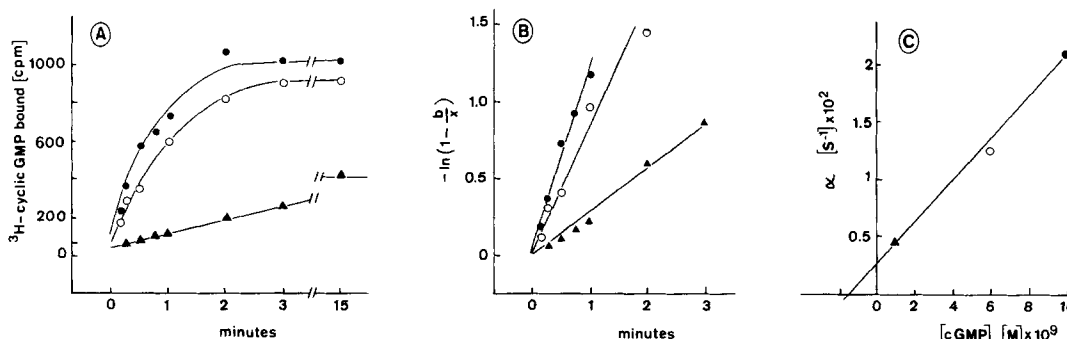


FIGURE 1 Determination of the rate constants by detection of binding before equilibrium is reached. (A) Binding is followed after mixing homogenate and different concentrations of ^3H -cGMP. (●), 10^{-8} M ; (○), $6 \times 10^{-9} \text{ M}$; (▲), 10^{-9} M . Aspecific bind-

ing was determined by including 10^{-5} M cGMP in the incubation mixture and are for: (●), 110 cpm; (○), 80 cpm; and (▲), 40 cpm. At 15 min, equilibrium was reached. (B) Representation of the data of A. From all data the appropriate blanks are subtracted and divided by the cpm bound under equilibrium conditions (15-min incubation). This yields b/x . Symbols as in A. (C) Representation of the data of B. The slope (α) of the curves in B is expressed vs. the cGMP concentrations, yielding a slope equal to k_1 , an intercept on the ordinate equal to k_{-1} , and an intercept on the abscissa equal to $-K_d$.

where y is the fraction of binding proteins occupied with ^3H -cGMP immediately after dilution or addition of an excess of unlabeled cGMP. In Fig. 2A the dissociation of ^3H -cGMP from the binding protein is shown after the addition of an excess of unlabeled cGMP. New associations between ^3H -cGMP and the protein are neglectable after addition of excess of unlabeled cGMP. Representation of the data of Fig. 2A as the left part of equation (5) vs. time results in a straight line (Fig. 2B) with a slope $k_{-1} = 4 \times 10^{-3} \text{ s}^{-1}$. A 100-fold dilution in buffer with or without 10^{-5} M cGMP gave identical results, indicating the absence of cooperativity at the rate constant of dissociation (6, 7).

In all experiments the filters were washed with buffer which requires about 10–15 s. Dissociation of the complex is sufficiently slow to allow this washing procedure.

Dissociation Constant

Determination of the dissociation constant with a Scatchard plot (36) is only allowed if equilibrium is reached. As can be seen from Eq. 3 and 4, this is determined by the time constant α , which depends on both rate constants and the cGMP concentration. By making use of the rate constants determined in Figs. 1 and 2, 95% of the equilibrium value is reached at 10^{-9} M cGMP after $\sim 10 \text{ min}$ and at 10^{-10} M cGMP after $\sim 20 \text{ min}$. A Scatchard plot of a 1-h incubation is shown in Fig. 3, yielding a dissociation constant of $K_d = 1.4 \times 10^{-9} \text{ M}$ and 50 fmol bound/homogenate of 10^7 cells (equal to 3,000 binding sites/cell = 10^{-8} M mean intracellular concentration). If the incubation time is 10 min, the number of binding proteins will be overestimated and the magnitude of the dissociation constant underestimated (reference 2 and unpublished observations). The rate constants derived from several experiments in vitro at 0°C (Figs. 1, 2, and 3) are $k_1 = 2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 3.5 \times 10^{-3} \text{ s}^{-1}$, $K_d = 1.4 \times 10^{-9} \text{ M}$, and 3,000 binding sites/cell.

Computer Simulations

The basal cGMP level is $\sim 1 \text{ pmol}/10^7 \text{ cells}$ (25) which corresponds to a mean concentration of $\sim 2 \times 10^{-7} \text{ M}$ (taking a cell diameter of $10 \mu\text{m}$). If this cGMP and the cGMP binding proteins are in the same compartment, equilibrium of binding will be reached in unstimulated cells, resulting in an occupancy of this binding protein ($K_d = 1.4 \times 10^{-9} \text{ M}$) of $\sim 99.3\%$. Since this is unlikely, either the K_d in vivo is much higher (K_d might depend on protein concentrations which are much lower in

vitro than in vivo) or basal cGMP and binding proteins are in different compartments. In the computer simulation experiments, we assume that basal cGMP and cGMP binding proteins are in different compartments and that cGMP accumulates only in the compartment of the cGMP binding proteins. We also assume that the cGMP peak has a triangle-like shape with a maximum at 10 s and a recovery of basal levels (0 M in this compartment) at 25 s after chemotactic stimulation (25) (Fig. 5 D). Intracellular occupation of the cGMP binding proteins is then given by the following differential equation:¹

$$\frac{db}{dt} = k_1([cGMP] - [R]_0b)(1 - b) - k_{-1} \cdot b \quad (6)$$

where $[R]_0$ is the total binding protein concentration (10^{-8} M) and $[cGMP]$ is the total cGMP concentration, given by the triangle of the cGMP accumulation and decline.

The fraction of binding proteins occupied by cGMP (b) was calculated with a PDP 11-45 computer by integration of Eq. 6 with 2-ms steps; negative free cGMP concentrations were excluded.

Binding of cGMP to its binding protein reaches a maximum at ~20 s after initiation of the cGMP peak, whereafter dissociation of the complex becomes noticeable.

The fraction of binding proteins occupied with cGMP depends strongly on the magnitude of the cGMP accumulation (Fig. 4 A). Variation of the rate constant of association (k_1) at fixed cGMP accumulation (Fig. 4 B) results in identical curves as variation of the cGMP accumulation at fixed values of k_1 (Fig. 4 A). This is evident from Eq. 6 where k_1 and $[cGMP]$ are multiplied. The magnitude of k_1 determines the sensitivity of the receptor; it determines the level to which cGMP must rise to reach a 50% occupation of the binding proteins.

The rate constant of dissociation (k_{-1}) has minor effects on the maximum of binding protein occupancy but pronounced effects on the length of the period the binding-protein-cGMP complex exists (Fig. 4 C). Since cGMP itself disappears faster than the complex, such a binding protein may be conceived as

¹ The mathematics of chemical reactions deal with concentrations of the components of the reaction. This introduces three problems which are difficult to solve. Firstly, although compartmentalization of cyclic GMP and cyclic GMP binding proteins may exist, the sizes of the compartments are unknown. In the absence of a better alternative, we take the volume of a whole cell (5×10^{-13} l) as the volume of the compartment which contains the cyclic GMP binding proteins and the cyclic GMP which accumulates after stimulation. Secondly, concentration (more precisely, activity) is a property based on high numbers of molecules in a relatively large volume. In the absence of a better alternative, we have to assume that the activity of 300 molecules (cyclic GMP or cyclic GMP binding proteins) in a volume of 5×10^{-13} l in a medium which differs from water and which changes with time is equal to a concentration of 10^{-9} M. Thirdly, a cyclic GMP molecule which is synthesized and released by the guanylate cyclase has no immediately equal changes to be present at all places in the compartment. A cyclic GMP binding protein in the immediate surroundings of the guanylate cyclase may have a higher chance to bind the cyclic GMP molecule than a binding protein localized at some distance from the guanylate cyclase. In the extreme case of very close approximation of the guanylate cyclase and the binding protein and a slow diffusion, this may imply that a cGMP molecule does not even come free, because it may have a higher probability to get bound than to reach the bulk of intracellular liquid (37). Since we have no information on the localization of guanylate cyclase and cyclic GMP binding proteins nor on the diffusion constant of intracellular cyclic GMP, we can only put forward the problem but we are in no position to solve it.

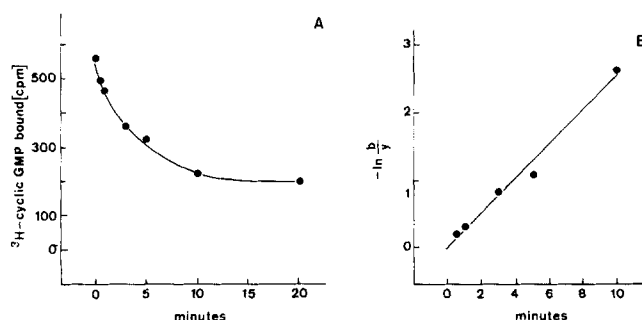


FIGURE 2 Determination of the rate constant of dissociation. (A) 2×10^{-8} M $[8\text{-}^3\text{H}]\text{cGMP}$ and homogenate were allowed to reach equilibrium; at $t = 0$ s, 10^{-5} M unlabeled cyclic GMP was added and dissociation of $[8\text{-}^3\text{H}]\text{cGMP}$ was measured. Aspecific binding was determined by including 10^{-5} M cGMP in the incubation mixture from the beginning and equals ~200 cpm. (B) Representation of the data of A. From all data, 200 cpm was subtracted and divided by the binding at $t = 0$. This yields b/y . The slope equals the rate constant of dissociation.

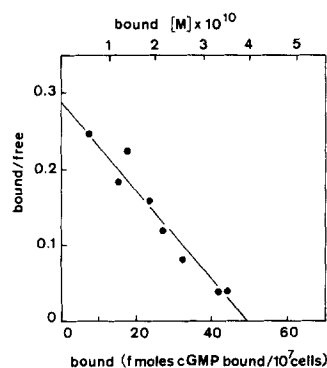


FIGURE 3 Determination of the dissociation constant and the total number of binding proteins. Homogenate and different concentrations (10^{-10} – 10^{-8} M) of $[8\text{-}^3\text{H}]\text{cGMP}$ were incubated for 1 h. The slope equals $-K_d$ and the intercept at the abscissa equals the total number of binding proteins; 50 fmol/ 10^7 cells = 3,000 molecules/cell = 9.5×10^{-9} M (taking a cell diameter of 10 μm and an equal distribution inside the cell).

the most simple form of memory for which k_{-1} determines the length of the information storage.

The dissociation constant ($K_d = k_{-1}/k_1$) does not give much information on receptor occupancy if the conditions are out of equilibrium. In Fig. 5, binding of cGMP to three binding proteins with identical dissociation constants is shown. Due to their differences in rate constants, the sensitivity and duration of binding are completely different.

Fig. 4 A shows that cGMP levels have to rise only to a small extent (3×10^{-8} M) to reach half-maximal receptor occupancy. This increase would be comparable with a cGMP accumulation in whole cells from 1.0 to 1.1 pmol/ 10^7 cells. In aggregative cells, this is evoked by stimulation with $\sim 10^{-10}$ M cAMP.

In Vivo Experiments

The foregoing results suggest that, in vivo, the cGMP binding proteins get occupied at very small increments of cGMP levels, and that occupancy is transient with a maximum at ~20–25 s after chemotactic stimulation and with a half-life of ~3–4 min. An attempt was made to show this in vivo by stimulation of aggregative cells with cAMP, rapid disruption of the cells, and fast determination of the number of binding proteins that can still be occupied with ^3H -cGMP.

The mathematics of these experiments are as follows: Assume that in vivo net-dissociation of the native complexes starts at $t = a$, and that, at that moment, the fraction of binding proteins occupied with native cGMP equals n , then the fraction

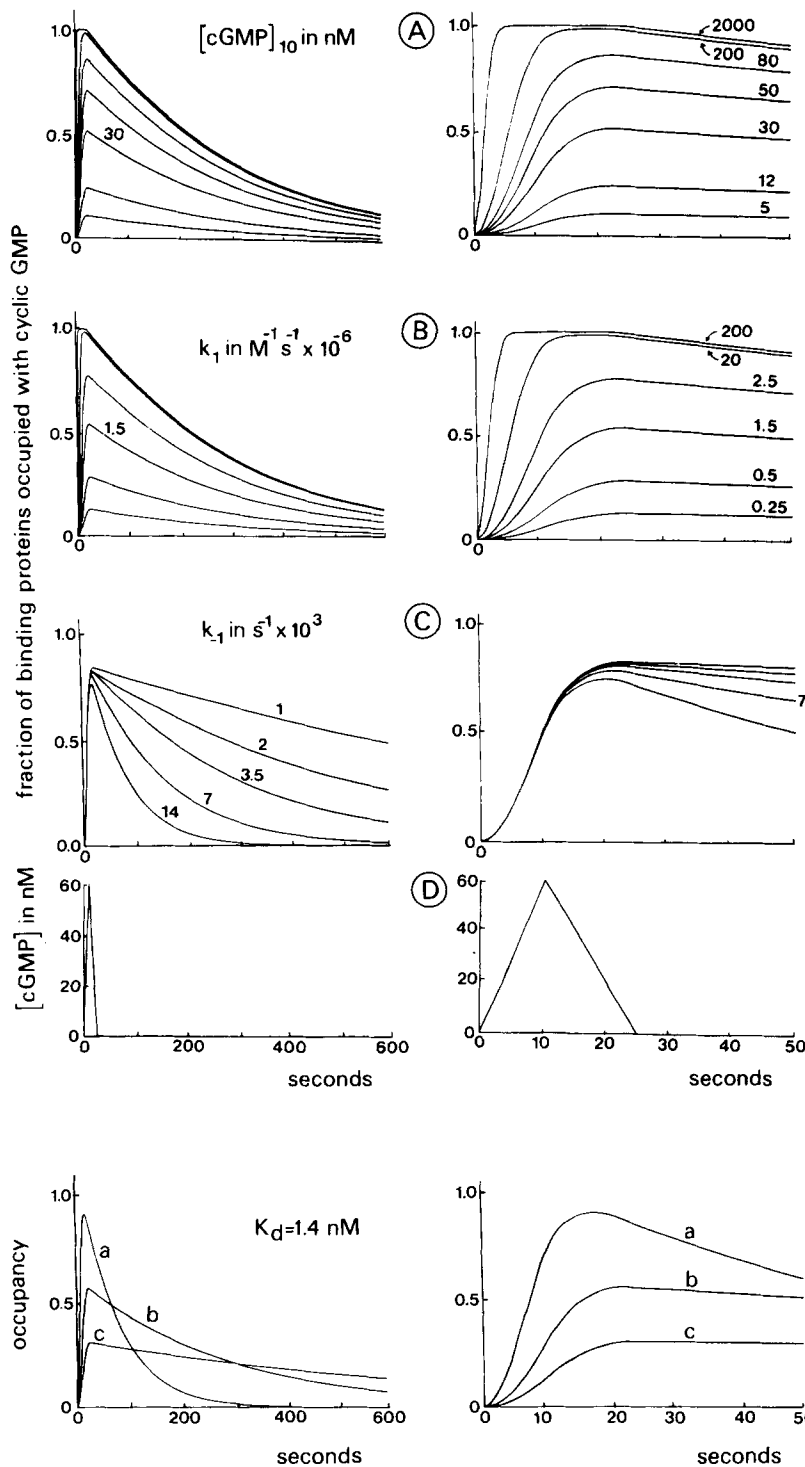


FIGURE 4 Calculation of receptor occupancy during a transient elevation of cGMP levels. Receptor occupancy is calculated with a computer program solving Eq. 9. Data are given for two time scales: 10 min (left), 50 s (right). (A) Variation of the top of the cGMP peak at fixed values of k_1 and k_{-1} . $[R]_0 = 10^{-8}$ M, $k_1 = 2.5 \times 10^6$ M $^{-1}$ s $^{-1}$, $k_{-1} = 3.5 \times 10^{-3}$ s $^{-1}$. (B) Variation of the rate constant of association at fixed values of $[cGMP]_{10}$ and k_{-1} . $[R]_0 = 10^{-8}$ M, $[cGMP]_{10} = 6 \times 10^{-8}$ M, $k_{-1} = 3.5 \times 10^{-3}$ s $^{-1}$. (C) Variation of the rate constant of dissociation at fixed values of $[cGMP]_{10}$ and k_1 . $[R]_0 = 10^{-8}$ M, $[cGMP]_{10} = 6 \times 10^{-8}$ M, $k_1 = 2.5 \times 10^6$ M $^{-1}$ s $^{-1}$. (D) The shape of the cGMP peak.

of binding proteins occupied with native cGMP in vivo (b) at different times thereafter is given by:

$$\ln \frac{b}{n} = -k_{-1}^v(t - a) \quad (7)$$

where k_{-1}^v is the rate constant of dissociation in vivo. At $t = t^s$, cells are sonicated and incubated with ^3H -cGMP. Dissociation of the native complex proceeds further, but now with the rate constant of dissociation in vitro (k_{-1}^0). The fraction of binding proteins occupied with native cGMP (bf) at the moment of filtration ($t^s + 72$) is given by:

$$\ln \frac{bf}{n} = -k_{-1}^v(t^s - a) - k_{-1}^0 \cdot 72 \quad (8)$$

The cGMP concentration in the binding assay (2×10^{-8} M ^3H -cGMP and $\sim 10^{-8}$ M native cGMP) is sufficient to label at least 95% of the binding proteins not occupied with native cGMP within 1 min. If we assume that binding proteins in nonstimulated cells are not occupied with native cGMP, then it follows that:

$$bf = 1 - \frac{\text{cpm} - b_l}{\text{cpm}^* - b_l} \quad (9)$$

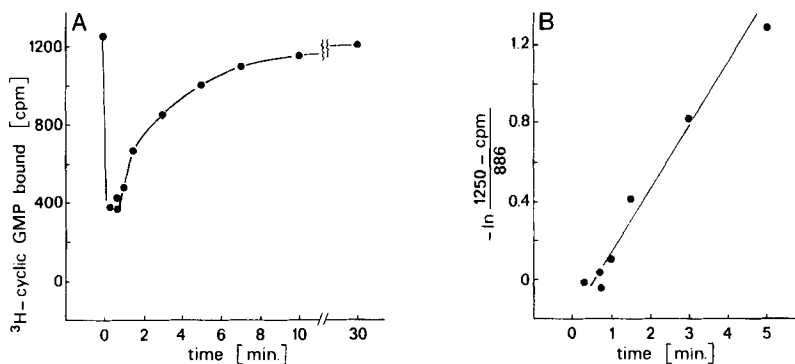


FIGURE 6 Dissociation of the cGMP binding protein complex in vivo. (A) Aggregative cells were stimulated with 10^{-7} M cyclic AMP at $t = 0$ s. At the time indicated ($t = t^s$ s), cells were homogenized by sonication for 6 s. At $t = t^s - 7$ s, DTT was added, and at $t = t^s - 5$ s, cells were placed in ice. Immediately after sonication homogenates were incubated in triplicate with $[^3\text{H}]\text{cGMP}$ for 1 min, followed by filtration. (B) Determination of the rate constant of dissociation in vivo (k_{-1}^s). As will be shown in Fig. 7 B, stimulation with 10^{-7} M cAMP leads to complete occupation of the binding protein at 30 s after stimulation, which indicates that

in Eq. 8, $n = 1$. Substitution of $cmp^* = 1,250$, $bl = 110$, and $k_{-1}^s = 3.5 \times 10^{-3} \text{ s}^{-1}$ in Eq. 8 and 9 yields:

$$-\ln \frac{1250 - \text{cpm}}{886} = k_{-1}^s (t^s - a)$$

where cpm is derived from A. Presentation of the left hand of this equation vs. the time of sonication (t^s) yields a line with a slope equal to the rate constant of dissociation in vivo ($k_{-1}^s = 6 \times 10^{-3} \text{ s}^{-1}$), and with an intercept on the abscissa equal to the time at which net-dissociation starts ($a = 30$ s).

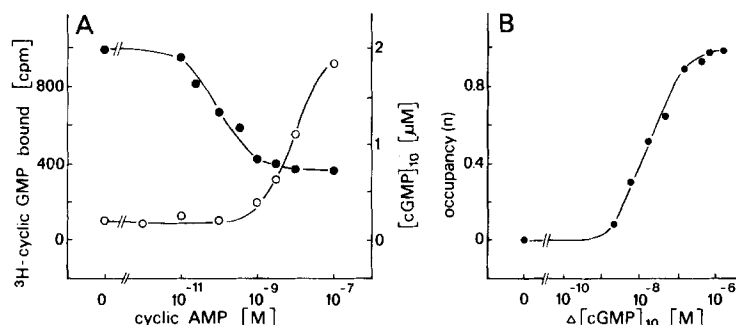


FIGURE 7 Dose-response curves of the chemoattractant-mediated binding of cGMP to its intracellular binding protein in vivo. (A) Aggregative cells were stimulated with different cAMP concentrations at 0 s. At 10 s, a sample of the cell suspension was withdrawn for the determination of the cGMP levels (O). At 33 s, 2 mM DTT was added; at 35 s, cells were placed in ice; and from 40 to 46 s cells were homogenized by sonication. Immediately after sonication, triplicate samples of the lysates were incubated with $[^3\text{H}]\text{cGMP}$ for 1 min, followed by filtration. (B) Data from A were transformed by calculation of the cGMP accumulation at 10 s after stimulation with Eq. 10, and by calculation of the occupancy of the binding protein in vivo at 30 s after stimulation (n) with Eq. 8 and 9. Substitution of $a = 30$, $k_{-1}^s = 6 \times 10^{-3}$ (both derived from Fig. 6 B), $k_{-1}^s = 3.5 \times 10^{-3}$, $t^s = 40$, $cmp^* = 1,000$, and $bl = 110$ in Eq. 8 and 9 yields

$$n = \frac{1,000 - \text{cpm}}{890} e^{0.31}$$

where cpm is derived from data (●) of Fig. 7 A.

where cpm is radioactivity bound to the lysate of stimulated cells, cmp^* is radioactivity bound to the lysate of nonstimulated cells, bl is blank: radioactivity was bound in the presence of 10^{-6} M cGMP. In Fig. 6A, cells were lysed at different times after stimulation of the cell suspension with a high cAMP concentration. Lysates prepared at 30–40 s after stimulation bind significantly less $[^3\text{H}]\text{cGMP}$ than lysates of nonstimulated cells, or lysates prepared at longer times after stimulation with cAMP. Stimulation with 10^{-6} M 5'AMP, or addition of 10^{-7} M cAMP after homogenization of nonstimulated cells, was without effect (data not shown). Fig. 6B is a linearization of the data of Fig. 6A using Eq. 8 and 9, which reveals that dissociation in vivo at 22°C is a first-order process with k_{-1} equal to $6 \times 10^{-3} \text{ s}^{-1}$; net-dissociation starts at ~ 30 s after chemotactic stimulation.

In Fig. 7A, cells were lysed at 40 s after stimulation with different cAMP concentrations. At high cAMP concentrations, still $[^3\text{H}]\text{cGMP}$ can be bound to the lysate, most probably due to partial dissociation of the native complexes during the 1-min period of the in vitro binding assay. cAMP has a half-maximal

effect at $\sim 10^{-10}$ M. The increase of cGMP levels at 10 s after stimulation with cAMP (Fig. 7A) can be written as

$$\Delta[\text{cGMP}]_{10} = 1.8 \times 10^{-8} \frac{[\text{cAMP}]}{[\text{cAMP}] + 10^{-8}} \quad (10)$$

This equation is a mathematical description of the dose-response curve shown in reference 25 and in Fig. 7A. The occupancy of the cGMP binding protein at 30 s after chemotactic stimulation (n) is calculated with Eq. 8 and 9. Presentation of n vs. $\Delta[\text{cGMP}]_{10}$ (Fig. 7B) reveals that the binding protein is half-maximally occupied by a cGMP accumulation of 2×10^{-8} M, which is consistent with the computer simulations (Fig. 4A and B) if k_1 in vivo at 22°C equals $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

DISCUSSION

Based on chromatographic data, Mato et al. (33) have shown three cGMP binding proteins in the soluble fraction of a

homogenate of *D. discoideum* cells. One of these proteins also binds cAMP (33) and other cyclic nucleotides (39) at very low concentrations. In most of the experiments, this binding protein could not be demonstrated and does not seem to be present in all species (33). Based on these results, we assume that binding of cGMP to this protein is nonspecific; it is absent in the described experiments. Figs. 1 and 2 do not reveal two specific binding proteins with different rate constants, nor do cGMP analogs show two specific binding proteins with different specificity (39). The two specific binding proteins which can be found after gel filtration (33) might be only different in size.

We determined the rate constant of association (k_1) and the rate constant of dissociation (k_{-1}) at 0°C in vitro by several methods, resulting in the following data: $k_1 = 2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 3.5 \times 10^{-3} \text{ s}^{-1}$, $K_d = 1.4 \times 10^{-9} \text{ M}$, with no signs of cooperativity (Figs. 1, 2, and 3). This value of the dissociation constant indicates that 50% of the binding proteins are occupied if the steady concentration of cGMP equals $1.4 \times 10^{-9} \text{ M}$. Since the basal mean cGMP concentration is about $2 \times 10^{-7} \text{ M}$, we assume the existence of two cGMP compartments. Compartment I contains all the cGMP of unstimulated cells, compartment II contains the cGMP-binding proteins and the cGMP which accumulates after chemotactic stimulation. In compartment II cGMP levels change only temporally after chemotactic stimulation. Binding of cGMP to the binding protein will not reach equilibrium. We combined the individual rate constants and the shape of the cGMP accumulation with the law of mass action and calculated the occupancy of this cGMP binding protein in nonequilibrium conditions. This reveals that not the dissociation constant (K_d) but the rate constant of association (k_1) determines the affinity of the binding protein for cGMP. After the destruction of free cGMP, its former presence is still noticeable, due to the binding proteins which are occupied with cGMP. The rate constant of dissociation determines not only the velocity at which this information is lost but also the time period after which new information can be stored. This time period extends over a few minutes. Autonomous oscillations of extracellular cAMP occur at a frequency of about one pulse per 5 min (14). This suggests that each pulse of extracellular cAMP can be recognized separately by the intracellular cGMP binding protein. Determinations of the occupancy of the binding protein in vivo are in good agreement with the results of the computer simulations. This has several implications:

(a) It is likely that there exist two cGMP compartments. Evidence for this has been provided by Pan and Wedner (34) and by Mato and Steiner (29) who showed with immunofluorescence techniques that cGMP is present mainly in the nucleus.

(b) The cGMP receptor in vivo can be described by $k_1 = 4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 6 \times 10^{-3} \text{ s}^{-1}$, cooperativity is absent and there are ~3,000 binding sites per cell which give a mean concentration of $\sim 10^{-8} \text{ M}$.

(c) The cGMP peak after chemoattractant stimulation can be described by a peak with the shape of a triangle, and its maximum at 10 s after stimulation, independent of the concentration of chemoattractant added.

(d) Binding of cGMP to the binding proteins is transient with a maximum at about 20–30 s after chemotactic stimulation, followed by a decay to basal levels with a half-life of ~2 min (Fig. 6).

(e) In aggregative cells, cGMP binding proteins get half-maximally occupied (at 30 s) at a cGMP accumulation produced by $1 \times 10^{-10} \text{ M}$ cAMP (Fig. 7). At this extracellular

cAMP concentration, intracellular cGMP levels increase from $2.0 \times 10^{-7} \text{ M}$ to $2.2 \times 10^{-7} \text{ M}$. This accumulation cannot be measured accurately. Therefore the absence of a detectable change of cGMP concentration cannot be used as evidence against a second messenger function of cGMP. Thus, although cGMP levels do not change measurably in vegetative cells after stimulation with cAMP, the levels may change sufficiently to cause a significant occupancy of cGMP binding proteins. On the other hand, during chemotaxis of aggregative cells towards cAMP, only a small number of the cell-surface cAMP-receptors are occupied (13, 27). This results in only a small increase of cGMP levels ($\Delta[\text{cGMP}]_{10} \approx 4 \times 10^{-8} \text{ M}$). The absence of

TABLE I
Possible Functions of the cGMP-binding Protein as Mediator of Chemoattractant-induced Cellular Responses

Cellular response	Source
<i>Mediated by the cGMP-binding protein *</i>	
PDE-induction by cAMP	Occurs in vegetative cells (18, 38).
PDE-induction by folic acid	Half-maximal PDE-induction occurs at $3 \times 10^{-8} \text{ M}$ (unpublished results), while half-maximal cGMP accumulation occurs at 10^{-5} M (31).
Light scattering by cAMP	Occurs in vegetative cells and in aggregative cells at low concentrations, 50% response at $\sim 10^{-9} \text{ M}$ (12).
pH response by cAMP	Occurs in aggregative cells at low concentrations, 50% response at $3 \times 10^{-10} \text{ M}$ (22).
Chemotaxis to cAMP	At threshold concentrations (10^{-9} M in aggregative cells, and 10^{-6} M in vegetative cells), only a small number of cAMP receptors are occupied (13, 27).
<i>Not mediated solely by this cGMP-binding protein†</i>	
Refractoriness by cAMP	Occurs at high cAMP concentrations (25). 50% refractoriness of the cGMP accumulation occurs by stimulation with 10^{-8} M cAMP (unpublished results).
Relay by cAMP in aggregative cells	50% response occurs at 10^{-7} – 10^{-8} M (9).
Phospholipid methylation by cGMP in vitro	Occurs at high cGMP concentrations, 50% activation at $8 \times 10^{-6} \text{ M}$ cGMP (1).§
Protein methylation by cAMP	Occurs only at high cAMP concentrations (28).

* The half-maximal response occurs at a concentration of chemoattractant which is equal to or below the concentration which results in half-maximal occupation of the cGMP binding proteins.

† The half-maximal response occurs at concentrations far above the concentration which induces 50% occupation of the cGMP binding protein. These responses may be mediated by cGMP, but not solely via the cGMP binding protein described in this paper. A cellular process in the second group can be involved in a process of the first group if only 1% of the second response is already sufficient to get 50% of the first group response, e.g. if protein methylation is involved in chemotaxis, 1% of the maximal increase of methylation should be sufficient to get chemotaxis.

§ This high concentration seems incompatible with a function during chemotaxis, since only very small increases of cGMP levels take place. However, if the methyltransferase works in the immediate environment of the guanylate cyclase and if diffusion of cGMP is limited, a high local cGMP concentration may be present (37). Stimulation of aggregative cells with low concentrations of cAMP should then activate methyltransferase significantly, without producing a high increase of mean cGMP levels.

chemotaxis towards cAMP in the mutant AGIP 55 (26) cannot be explained solely by the decrease of production of cGMP, because cGMP levels can still increase up to 10^{-6} M in this mutant (26).

(f) The cellular functions which are evoked by chemoattractants and are mediated by this cGMP protein take place at very low accumulations of cGMP levels. In aggregative cells, 50% of the response should be evoked by extracellular cAMP at a concentration of 10^{-10} – 10^{-9} M and not at a concentration where 50% of the cell surface cAMP receptors are occupied (between 10^{-7} and 10^{-8} M). Even in vegetative cells, sufficient cAMP receptors might be present to produce enough cGMP to reach significant occupancy of the cGMP binding proteins. The cellular response which might be mediated by this binding protein and responses which can not be mediated solely by this binding protein are listed in Table I.

The function of this cGMP binding protein is unknown. No cGMP-dependent protein kinase activity has been demonstrated in *D. discoideum* (33, 35).

The kinetics of binding and of the cGMP accumulation show that 50% of the cGMP binding proteins get occupied at a very low occupancy of cell surface cAMP receptors. This limits the cellular responses which can be mediated solely by this receptor to PDE-induction, light scattering, pH changes, and chemotaxis.

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